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Award Number: W81XWH-09-1-0399

TITLE: Mechanism of Prostate Cancer Prevention by Down-Regulation of the GH/IGF Axis

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REPORT DATE: Sep 2013

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Sep 2013	Final	1 July 2009 – 30 June 2013

4. TITLE AND SUBTITLE

Mechanism of Prostate Cancer Prevention by Down-Regulation of the GH/IGF Axis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0399

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Illinois
Chicago, IL 60612-4305

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this project was to test the hypothesis that growth hormone (GH) stimulates specific pathways, some of which are independent of IGF-I, for promoting proliferation and inhibiting death in prostate cancer cells. The first aim was to determine which of the multiple signaling pathways stimulated by GH receptor are required to promote prostate cancer. Our strategy was to cross mice that develop prostate cancers due to a large T antigen (TAg) transgene with mice that lack discrete segments of the intracellular portion of the GH receptor. We have not yet completed this experiment due to insufficient breeder fecundity but the studies should be complete by October of this year. To assess the relative contribution of IGF-I and GH to prostate carcinogenesis, we grafted prostate tissue harboring the TAg transgene. The grafts were either Ghr+/+ or Ghr-/- and therefore were able to respond to IGF-I but not detect GH. Our results suggest that IGF-I is the major driver of carcinogenesis. We also planned to propagate human prostate cancer cells in vitro and expose them to a human growth hormone antagonist. In vitro, however, the cells were neither stimulated by recombinant human GH nor inhibited by GH antagonist. Studies GH antagonist mice demonstrated that the presence of a GH antagonist can significantly retard the progression of prostate carcinogenesis driven by the powerful SV40 oncogene.

15. SUBJECT TERMS
Growth hormone, growth hormone antagonist, prostate cancer, cancer prevention.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

It has been known for many years that androgen ablation can reverse the course of the prostate cancer and this has formed the foundation of therapy for decades. Invariably, however, castrate resistant forms emerge that resume disease progression. In recent years, additional pathways have been identified that may provide alternative targets to androgen signaling. One such pathway involves the growth hormone/insulin-like growth factor-I (GH/IGF-I) axis. In vitro and in vivo studies of rodent and primate model systems illustrate that GH and IGF-I can induce prostate epithelial cell proliferation and differentiation while blocking apoptosis. Recent clinical trials indicate that elevated circulating IGF-I confers an increased risk for the development of prostate cancer. Our hypothesis was that GH stimulates specific pathways, some of which are independent of IGF-I, for promoting proliferation and inhibiting apoptosis in prostate cancer cells. Our first aim was to determine what signaling pathways stimulated by GH and its receptor are required to promote prostate cancer. We crossed the C3(1)/TAg mouse, which develops prostate cancers, with mice that have defined deletions in their GH receptor that stimulate specific signaling pathways. The goal of these experiments was to get learn which GH-stimulated pathways are most important in supporting carcinogenesis. Due to the lack of fecundity of the breeder mice, however, we have not been able to generate the number of bigenic mice proposed in the original application. These mice will all be sacrificed by September 11, 2013. At that time, all prostate tissues will be harvested, processed and analyzed for histopathology. Another experiment in Aim 1 was to compare the relative contributions of GH and IGF-I to tumor growth. We generated mice that harbored one copy of the TAg transgene and were homozygous for either the wild-type ($Ghr^{+/+}$) or knockout ($Ghr^{-/-}$) of the GH receptor. Prostates of these mice were then transplanted into immunodeficient mice where they could grow in the presence of normal physiologic GH and IGF-I serum titers. We observed that prostate carcinogenesis proceeded similarly in the $Ghr^{-/-}$ prostates as in the $Ghr^{+/+}$ prostates indicating that IGF-I signaling is the dominant pathway driving carcinogenesis in this system. Our second aim was to determine which pathways are involved in cancer regression caused by GH removal or antagonism. We had originally planned to use the GH antagonist developed by our collaborator, Dr. John Kopchick, to study the mechanisms that GH antagonists can kill prostate cancer cells resulting in tumor regression. We discovered, however, that the prostate cancer cell lines LNCaP and PC-3 were both insensitive to either GH stimulation or antagonism as judged by *in vitro* cell proliferation assays. To circumvent this problem, we received permission from the CDMRP to conduct an *in vivo* experiment in which mice harboring a transgene for the bovine GH antagonist (GHA mice) were crossed with the C3(1)/TAg mice. The results indicate that GHA mice had significantly fewer preneoplastic lesions. In summary, the results of this study suggest that IGF-I, rather than GH, is the most important driver of prostate carcinogenesis in this system.

BODY

Below is a summary of results for each Task listed in our Statement of Work along with comments on problems encountered and results gathered.

Task 1: To determine what signaling pathways stimulated by GH and its receptor are required to promote prostate cancer.

The specific action items were:

a. *Animal protocol reviewed (ACURO; months 1 - 2).*
b. *Cross C3(1)/TAg mice with GHR mutant mice (months 3 - 18).*
c. *Conduct PCR analysis of mouse tail snips for genotyping (months 3 - 18).*
d. *Sacrifice mice for necropsy and histology of prostate glands (months 18 - 26).*
e. *Histologic analysis of slides, measurement of prostate lesions (months 26 – 29).*
f. *Data analysis and report writing (months 29 - 36).*

The animal protocol was approved by our IACUC at the beginning of the funding period and has been renewed and extended through 2015 (UIC ACC approval number 11-216). We obtained the GHR knockin mice from Dr. Michael Waters of the University of Queensland, Australia and completed the initial cross as presented in Table 1. During previous reporting periods, we noted difficulty generating homozygous GH receptor mutant mice for the 391 and 569 mutants. These difficulties were resolved by increasing the cohort of breeders, which was a time-consuming process. The mice described in Table 1 have been generated and bred to produce our experimental animals as outlined in Table 2.

Table 1. Initial C3(1)/TAg x GHR Cross
Female (T/t + G/G) x Male (T/T + g/g)

		Genotype		
Class	Proportion	TAg		GHR
A	1/2	T/T	+	G/g
B	1/2	t/T	+	G/g

Female C3(1)/TAg mice heterozygous for the transgene were crossed with GHR mutant mice. The pups were used in the C3(1)/TAg / GHR hybrid production cross. T: lacking C3(1)/TAg; t C3(1)/TAg; G: GHR/BP wild type; g: GHR/BP null

Table 2. C3(1)/TAg /GHR Mutant Hybrid Production Cross
t/T + G/g x T/T + G/g

		Genotype			
Class	Proportion	TAg		GHR	Animal Usage
A	1/8	T/T	+	G/G	
B	2/8	T/T	+	G/g	Breeding
C	1/8	T/T	+	g/g	Breeding
D	1/8	t/T	+	G/G	Males: + controls; females: breeders
E	2/8	t/T	+	G/g	Breeding
F	1/8	t/T	+	g/g	Experimental group

Pups used for the carcinogenesis studies were of the 'D' and 'F' class highlighted above. T: lacking C3(1)/TAg; t carrying C3(1)/TAg; G: GHR wild type; g: GHR mutant .

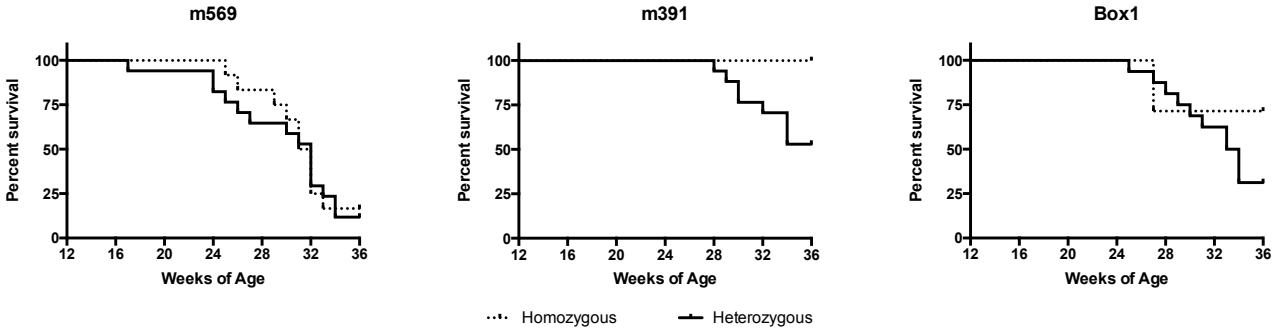
Table 3 is a summary of the experimental animals that were generated. Eleven of these animals are still on test and have not been sacrificed (final sacrifice date is scheduled for September 11, 2013). These include one mouse heterozygous and one homozygous for the m569 knockin, four mice homozygous and four mice heterozygous for the M391 knockin and one mouse that is homozygous for the Box1 knockin. These are the animals that will be used to address the question of which part of the GH receptor is associated with driving prostate carcinogenesis.

Table 3. Total number of test mice produced for Task 1.

M569		M391		Box1	
Homozygous	Heterozygous	Homozygous	Heterozygous	Homozygous	Heterozygous
15	17	10	17	7	16

We are disappointed with the yield of these crosses. We originally planned on generating 30 of each genotype, but due to poor fecundity, we were only able to produce one quarter to one half of that target number. Another major issue that we have faced is the survival of the mice we were able to generate. As illustrated in Figure 1 below, a substantial number of mice died prior to the end of the study at 36 weeks of age. This will require us to group the mice by their age for age-matched comparison of tumorigenesis. It has also forced us to keep the study open so that we have a reasonable chance at producing enough animals to detect a significant difference in prostate carcinogenesis among the groups. Once the last mouse is sacrificed on September 11, we anticipate that we will be able to process all tissues and perform the pathologic analysis within two to three weeks.

Figure 1. Survival curves for mice derived from classes D & F of Table 2 above.



Task 2: To compare the relative contributions of GH and IGF-I to tumor growth.

The specific action items were:

a. *Breed mice bearing TAg and either $Ghr^{+/+}$ (N=15) or $Ghr^{-/-}$ (N=15) (months 3 - 6).*
b. *Genotype pups by PCR (months 3 - 8).*
c. *Transplant 3-day-old prostates under kidney capsule of immunodeficient recipients (months 3 - 8).*
d. *Sacrifice hosts (months 11 - 17).*
e. *Process tissues and analyze lesions using image analysis software (months 18-24).*

We have completed all action items listed above. Prostates from one-week-old mice heterozygous for SV40 transgene and either homozygous for the wild-type GH receptor (*$Ghr^{+/+}$*) or the null for this gene (*$Ghr^{-/-}$*) (N=14 from each group) were transplanted under the kidney capsules of immunodeficient nude (*$Foxn1^{nu/nu}$*) mice. Figure 2 is a photomicrograph of kidneys from nude recipients (left) showing how two prostates were transplanted under the renal capsules of each kidney. As can be seen, the prostates thrived in this site. Figure 3 depicts a mouse prostate freed of surrounding connective tissue and ready for transplantation. Eighteen weeks after transplantation, the nude hosts were sacrificed and the transplanted prostates were fixed in formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) for assessment of neoplastic progression [1].

The sections of prostates from the two groups harbored a similar number of preneoplastic lesions (Figure 4 and Table 4 below). The sections were evaluated using the guidelines published by Cardiff and colleagues [2]. Both groups had prostatic epithelial cells with enlarged, hyperchromatic nuclei. Some sections from both groups revealed relatively small foci with one or two layers of atypical cells. The fibromuscular stroma was intact and the duct profile was normal. The cells were generally more columnar, larger, and taller than adjacent normal cells. They had abundant pale cytoplasm with hyperchromatic but minimally pleomorphic nuclei. However, there was no significant difference in the incidence or severity of preneoplastic lesions between the two groups at 5 % level (chi-squared test; Table 4).

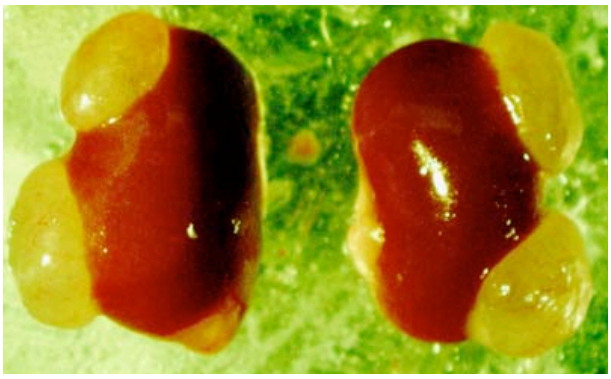
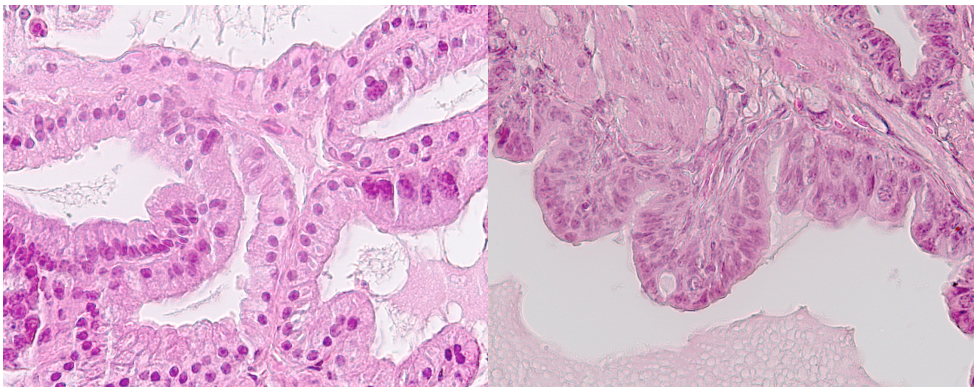


Figure 2. Prostates transplanted under the kidney capsules (2 per capsule) of each nude recipient.

Figure 3. A prostate ready for transplantation.



Ghr $^{+/+}$ *Ghr* $^{-/-}$

Figure 4. Photomicrographs of prostates transplanted from mice that were heterozygous for the oncogenic SV40 transgene and homozygous for wild-type *Ghr* or the GH receptor knocked out (*Ghr*$^{-/-}$). Note that both groups harbor areas of atypical hyperplasia.

Table 4. Absence of the GH receptor does not significantly impede progression of SV40-driven mouse prostate carcinogenesis.

	Ghr$^{+/+}$	*Ghr*$^{-/-}$
Adenocarcinoma	0	0
Carcinoma *in situ*	0	0
AH +++	2	2
AH ++	5	6
AH +	6	2
AH ±	1	4

The data represent the presence of multifocal, atypical hyperplasia (AH) at a frequency that is minimal (±), slight (+), moderate (++) or prevalent (+++).

Task 3: To determine what pathways are involved in cancer regression caused by GH removal or antagonism.

The modified action items were (CMDMRP-approved modifications were *f – i*):

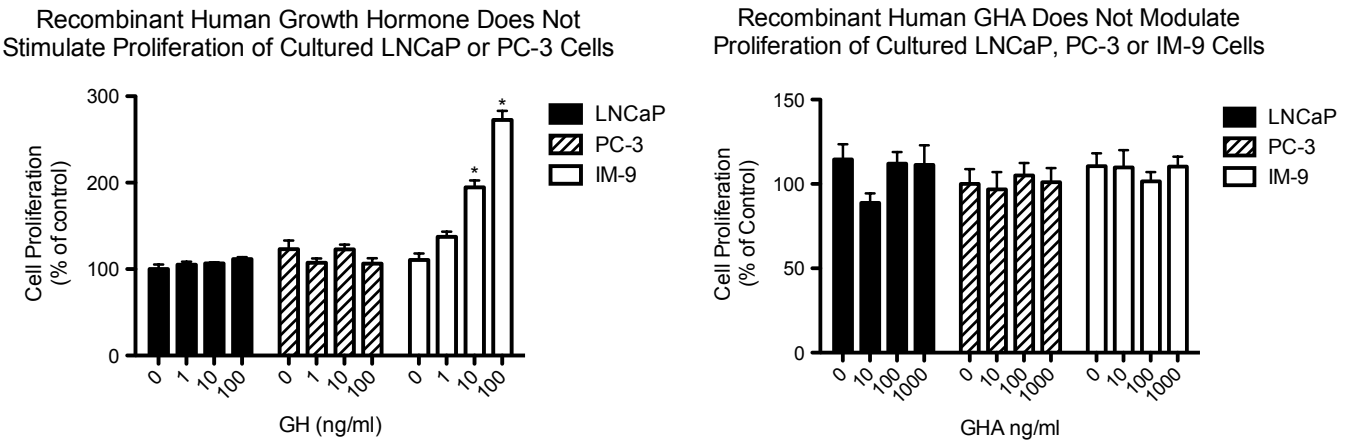
a. *Conduct site-directed mutagenesis of the human GH cDNA that changes the glycine codon at position 120 to one encoding lysine (months 3 - 6).*
b. *Purify human and mouse GH from inclusion bodies (months 6 - 36).*
c. *Scale up production of the E. coli cultures producing mouse and human GH antagonists (months 6 - 36).*
d. *Scale up purification of human and mouse GH antagonists from E. coli cultures (months 6 – 36). Deliverables are purified human and mouse GH antagonists for use in the proposed studies.*
e. *Treat human (LNCaP & PC-3) and mouse (Pr-117) prostate cancer cells with GHA in vitro (months 9 - 30).*
f. *Cross C3(1)/TAg mice with GHA mice heterozygous for the C3(1)/TAg oncogene and either heterozygous for the GHA transgene or null for this transgene (months 37 – 40).*
g. *Sacrifice mice (months 46 - 48).*
h. *Analyze prostate tissues for activity of GH related pathways (months 46 - 48)*
i. *Prepare report of results.*

Dr. Kopchick of Ohio University has provided us with recombinant human GH and recombinant human GHA (Task 3a through 3d). The GH and GHA were produced in *E. coli* utilizing an expression scheme similar to Sereikaite et al. [3]. Briefly, GH or GHA cDNA was cloned into the expression vector pET101 and transformed into BL21Star (Invitrogen), an *E. coli* strain specialized for high-level protein production. Under these conditions, protein production is induced by IPTG and accumulates in inclusion bodies made up of highly concentrated GH or GHA. The inclusion bodies were sonicated and centrifuged. Highly purified inclusion bodies were solubilized and steps were performed to allow the GH or GHA to refold to its native structure. Purification steps were conducted using procedures previously described by Patra et al. [4].

We have used GH and the GHA purified as described above to evaluate the sensitivity of LNCaP and PC-3 cells to GH or GHA (Task 3e). Human prostate cancer cell lines designated LNCaP and PC-3 and human lymphoblast cells designated IM-9 were purchased from the American Type Culture Collection, (Manassas, VA). The cells were propagated at 37 °C in 5% CO_2 in RPMI 1640 medium supplemented with fetal bovine serum (FBS, 10%) penicillin (100 units/ml) and streptomycin (100 µg/ml). Cells in log phase growth were harvested by pipetting (IM-9 cells) or by trypsinization (LNCaP and PC-3) followed by extensive washing to remove all traces of enzyme. Cells were resuspended in medium in which the FBS concentration was lowered to 1% and a total of 5,000 cells were seeded per well of 96-well clear, flat-bottom plates (Microtest 96®, Falcon). The cells were then treated with recombinant human GH (0 – 100 ng/ml RPMI 1640 medium), or recombinant human GH antagonist (0 – 1,000 ng/ml RPMI 1640 medium). The cells were incubated in triplicate in the presence of test substance or vehicle for 96 hours at 37 °C and evaluated for viability with a commercial absorbance assay that measured the amount of viable cells (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega Corp, Madison, WI). Activity was expressed as the percentage of viable cells present relative to the negative (RPMI 1640) control at each GH or GHA concentration. The positive control was treatment of IM-9 cells with GH. Data were analyzed by two-way ANOVA ($P \leq 0.05$, indicated by an asterisk) with the Bonferroni post test to compare the response on LNCaP or PC-3 to the human lymphoblast line IM-9, which is known to respond to GH stimulation *in vitro*.

The data presented below demonstrate that neither recombinant human GH nor the GHA significantly affect proliferation of either LNCaP or PC-3 cells propagated *in vitro*. We interpret these results as follows. Human LNCaP or PC-3 cells propagated in culture have been selected to be independent of GH. The serum used in laboratories around the world is non-primate serum (*e.g.,* bovine, equine or porcine). The GHs derived from these species are well known not to stimulate the human GH receptor. Thus, human cancer cells propagated in the most common forms of media proliferate in the absence of GH signaling and do not respond to recombinant human GH or GH antagonist.

Figure 5. Modulation of GH signaling does not affect proliferation of cultured LNCaP or PC-3 cells



To address this problem, we received approval from the CDMRP for the following experiments. Our co-investigator, Dr. John Kopchick, has made available to us mice that harbor a bovine GH antagonist transgene [5]. The transgene is the wild-type bovine GH sequence with three amino acid alterations in helix three: Q117L, G119R and A122D. The protein product of this mutated boGH can inhibit binding of [^{125}I]-GH to liver membrane preparations. Expression of the transgene is driven in a non-targeted fashion by the mouse metallothionein I transcriptional regulatory sequences. These mice are designated GH antagonist (GHA) mice and have decreased circulating IGF-I concentrations and exhibit a dwarf phenotype [5, 6]. The mechanism by which the GH antagonist acts is by failing to induce 'proper or functional' GH receptor dimerization [7].

To test the hypothesis posed in this Task, we crossed heterozygous C3(1)/TAg mice with mice that were heterozygous for the GHA transgene. Mice were evaluated by PCR for the presence of both the SV40 and GHA transgenes or SV40 without the GHA (each representing one fourth of the offspring; *i.e.*, approximately half of the total offspring were available for the study). Mice were housed singly to prevent fighting and sacrificed at 9 months of age. These experiments have allowed us to get around the issue of GH non-responsiveness in cultured cells. As presented in Figure 6, GHA mice weigh approximately 30% less than wild type mice at 36 weeks of age when all mice were sacrificed.

Figure 6. Male mice harboring the GHA transgene weighed approximately 30% less than wild-type mice at 32 weeks of age.

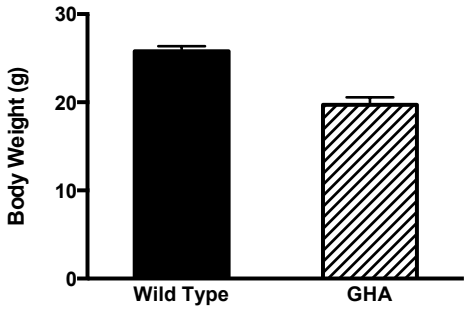


Figure 7. The survival of GHA mice was not significantly different from wild-type mice as judged by the log-rank (Mantel-Cox) test.

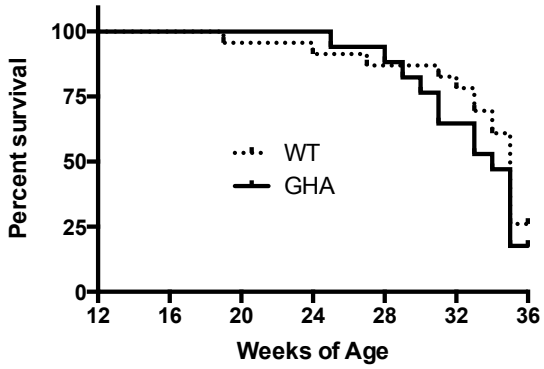
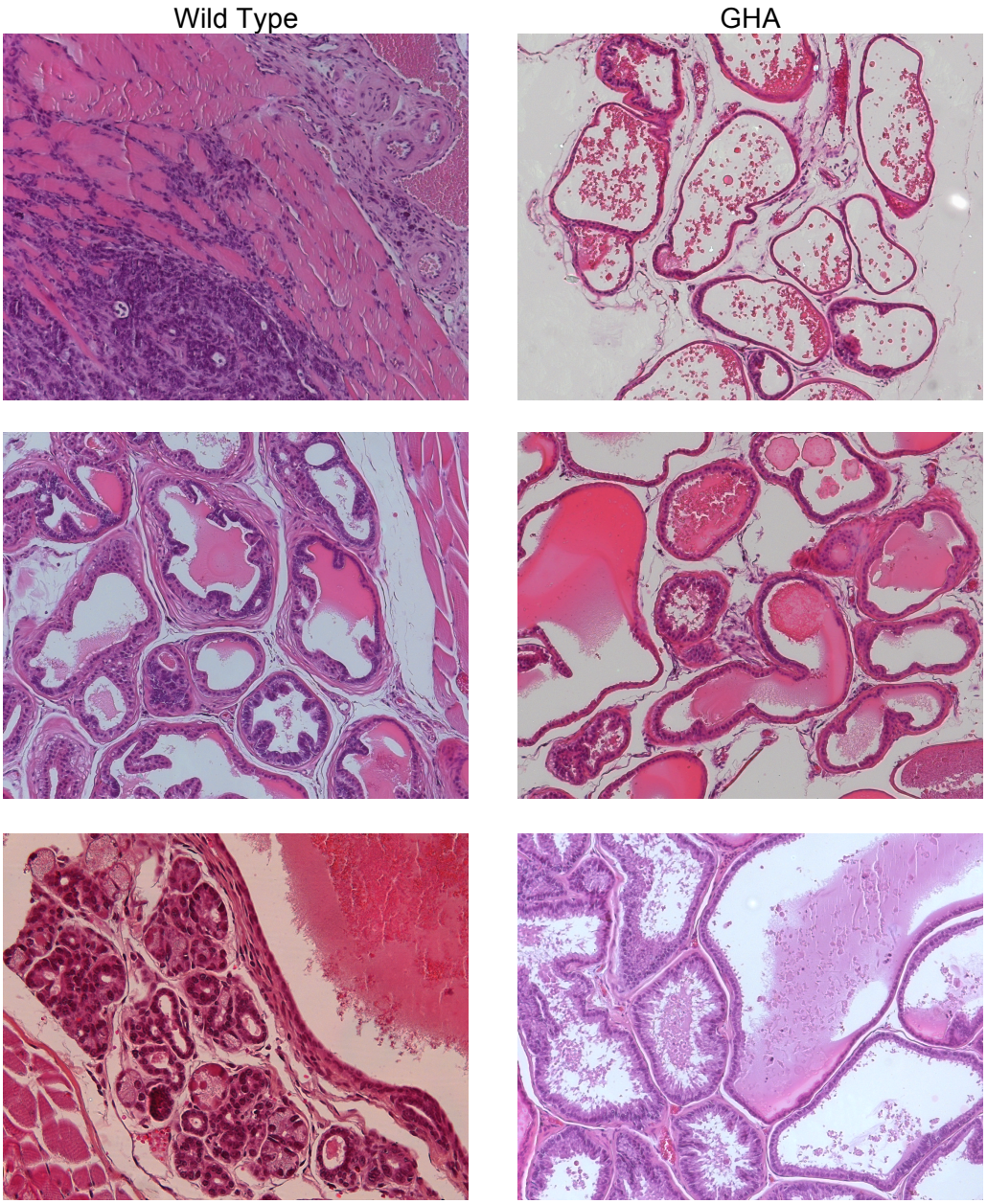


Table 5. The presence of the GHA transgene significantly decreased the number of multifocal atypical hyperplasia driven by SV40/TAg.

	Wild-type	GHA
Adenocarcinoma	1	1
Carcinoma *in situ*	2	2
AH +++	3	0
AH ++	4	4
AH +	7	3
AH ±	0	4

As presented in Table 7, there was substantial mortality in both groups (wild type N=17; GHA N=14) beginning around 32 weeks of age. Examples of prostate tissue histology are presented in Figure 8 below and summarized in Table 5. The abbreviation “AH” in Table 5 represents multifocal atypical hyperplasia with focal crowding of cells without papillary growth. Carcinoma *in situ* were characterized by atypical proliferations with papillary, microglandular or solid growth patterns, but without evidence of invasion. There were no differences between the two groups in the incidence of tumors [1/17 (6%) vs. 1/14 (7%)] and carcinoma *in situ* [2/17 (12%) vs. 2/14 (14%)] or the two combined [3/17 (18%) vs. 3/14 (21%)]. The p values were 1.00 (two-sided Fisher exact test). For the atypical hyperplasia, the chi square test and the Fisher exact test were used to evaluate the apparent downward shift from minimal (±) to slight (+), to moderate (++) to prevalent (+++) when comparing the wild type with the GHA prostates. Although the chi square test is not statistically valid because of low or zero values for some categories, there was a significant linear trend to a reduced severity of the atypical hyperplasia (p = 0.0239). When pooling all animals with scores of more than minimal (14 for wild type and 7 for GHA mice) and those of less than minimal (0 for wild type and 5 for GHA+ mice) the difference was significant (p=0.0286) when using a two-sided Fisher exact test.

Figure 8. Photomicrographs showing preneoplastic lesions and cancer in prostates of mice heterozygous for the SV40/TAg transgene either with (GHA) or without (Wild Type) the GH antagonist transgene.



KEY RESEARCH ACCOMPLISHMENTS

- We have generated three sets of mutant mice that are heterozygous for the SV40 oncogene and either homozygous or heterozygous for the specific GH receptor mutations. Analysis of the prostates of these mice in September should reveal important

information regarding the importance of specific signaling pathways for the progression of mouse prostate caner.

- The results from the experiments of Task 2 established that the progression of SV40-driven mouse prostate carcinogenesis is as rapid in prostate tissue lacking GH receptor as in tissue with intact, wild type GH signaling when normal physiologic levels of IGF-I are present.
- The results of experiments performed to complete Task 3 demonstrate that the presence of GH antagonist can significantly reduce the incidence of preneoplastic lesions driven by SV40 in the mouse prostate. Given that the serum IGF-I levels in the GHA mouse are intermediate between levels found in the GH receptor knockout mouse and mice with wild-type GH receptor [8], these findings are consistent with the results of Task 2, which suggest that IGF-I is a critical driver of carcinogenesis in this system.

REPORABLE OUTCOMES

- Invited symposium seminar presented at the 92nd Annual Meeting of the Endocrine Society [9].
- Invited symposium seminar presented at the 2011 IMPaCT Conference [10].
- Qi Shen, who was supported by this award, is now a Research Technician in the laboratory of Dr. Liqun Luo, Professor, Howard Hughes Medical Institute, Stanford University.

CONCLUSION

The major conclusion from the results obtained from this project are that IGF-I, rather than GH, is capable of supporting prostate carcinogenesis in the mouse. This major finding is supported by the experiments in which prostates of mice that harbor the SV40 oncogene, but lacking in GH signaling do to the absence of a functioning receptor, were able to progress to early stages of carcinogenesis as rapidly as SV40-containing prostates that had a normal, functional GH signaling. Since GH regulates IGF-I expression, when GH signaling is disrupted, IGF-I levels drop. In past work, we observed that whenever GH signaling was compromised, prostate carcinogenesis was impeded. However, we could not determine if this protective effect was do disruption of GH or IGF-I signaling. By transplanting the prostates to a immunodeficient host with normal IGF-I levels, were able to circumvent this problem.

Another major discovery was that an antagonist of GH action significantly retard prostate carcinogenesis. Based on our results from the transplantation experiments described above, it seems that the likely mechanism of action of GH antagonists is through inhibition of IGF-I production. Somavert, an FDA approved drug that antagonizes GH signaling, is known to significantly lower IGF-I serum titers in acromegaly patients. Somavert was developed from the GH antagonist used in the present studies. Therefore, somavert may prove useful in blocking prostate carcinogenesis in man as it has proven here to work in the mouse.

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